Bacillus subtilis: sporulation, competence and the ability to take up fluorescently labelled DNA

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Chapter 5

Visualizing and tracking down the uptake and integration of labelled DNA during competence of *Bacillus subtilis* by time-lapse microscopy

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Abstract

Bacillus subtilis is a naturally competent bacterium capable of taking up and integrating exogenous DNA from the environment. In this work we set up a system for real-time following of transformation of *B. subtilis* during competence. We show by use of a micro-fluidics system that *B. subtilis* can successfully transport, integrate and express fluorescently labelled DNA. Replacement of a specific locus on the chromosome was achieved and visualised. We demonstrate that cell division is not required for expression of integrated exogenous DNA.

Introduction

A fascinating adaptive strategy of *B. subtilis* is its ability to take up exogenous DNA from the environment during natural competence. This process of transformation occurs through initial binding of double stranded DNA to the competence machinery (Chung and Dubnau, 1998; Hahn et al., 1993; Inamine and Dubnau, 1995), after which one of the DNA strands is degraded and transported as single stranded DNA (ssDNA) through the ComEC channel (Draskovic and Dubnau, 2005; Londoño-Vallejo and Dubnau, 1993; Takeno et al., 2011). As the ssDNA enters the cells it is bound and protected by SsbA and SsbB (Baitin et al., 2008; Grove et al., 2005; Yadav et al., 2012, 2014). One of the main components responsible for the subsequent integration into the host chromosome of homologous DNA is RecA (Bell et al., 2012; Cox, 2007; Yadav et al., 2014). Several studies have been shown the co-localisation of components of the competence machinery, using fusions to fluorescent proteins (Hahn et al., 2005; Kaufenstein et al., 2011; Kramer et al., 2007) .

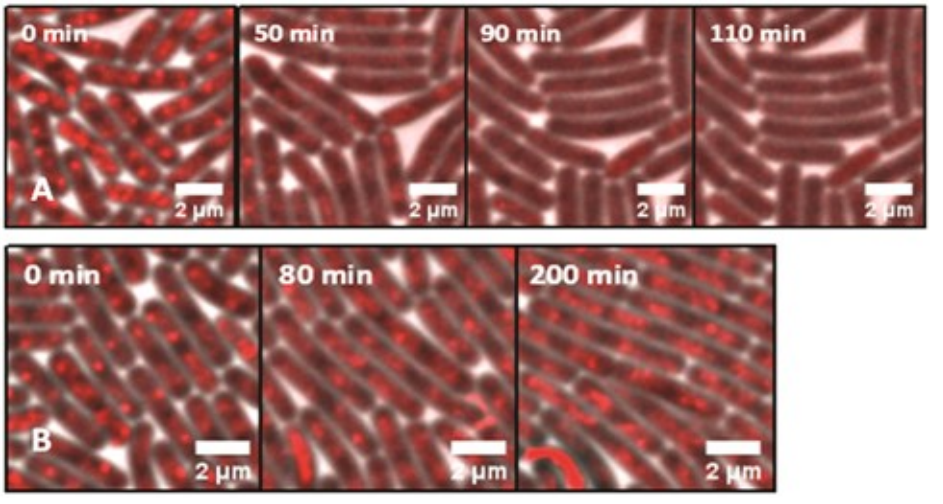
Previously, we tested several methods of fluorescent labelling of DNA in order to develop a way to directly visualise interactions of the incoming DNA with components of the competence machinery. We showed that labelled DNA interacts with components of the competence machinery such as ComFC as well as RecA and the chromosome (Chapter 4).

The ability of *B. subtilis* to take up fluorescently labelled DNA through the competence machinery creates the exciting opportunity to follow the transformation process in real time.The advances in microfluidics offer a technique by which conditions during time-lapse microscopy can be changed and more accurately controlled. By using labelled DNA in combination with microfluidics and time lapse microscopy it becomes possible to follow the entire transformation process from binding to uptake to integration and expression. Use of these methods may allow us to determine how long the integration process takes and how long it takes before the integrated exogenous DNA is expressed. Furthermore, it enables us to determine if cell division is required for expression of the integrated DNA. Here we use DNA covalently-labelled with DyLight650 or Fluorescein to visualise the exogenous incoming DNA. By using a ParB-GFP/mKate construct, which forms foci on a specific locus on the chromosome we determined life co-localisation and replacement of the focus by the exogenous DNA. The use of an antibiotic resistance gene in the labelled DNA allows for selection off successful transformants and the use of promoter *gfp* fusions allows us to determine if and when expression of the integrated DNA occurs.

Results

To determine whether replacement of a specific locus on the chromosome can be visualised we first performed a time lapse experiment with unlabelled DNA on polyacrylamide slides containing medium and 1mM of IPTG. *B. subtilis* 168_*amyE::*P$_{xyl}$-*comK*_*thrC::*P$_{spank}$-*parB-mkate* was transformed with pDG1664, which integrates into the *thrC* locus and should therefore be able to replace the *parB-mkate* construct. Because of the *parS* site present in the *parB* gene, this locus is visible as a red focus due to the fusion of *mkate* to *parB*. Samples were imaged every 10 minutes for a total time of 4 hrs. Approximately 30 minutes after addition of DNA the time-lapse experiment using a solid polyacrylamide slide containing competence medium and 1mM IPTG was started. A control containing no DNA was taken to ensure that loss of foci is indeed the result of integration of transformed DNA.

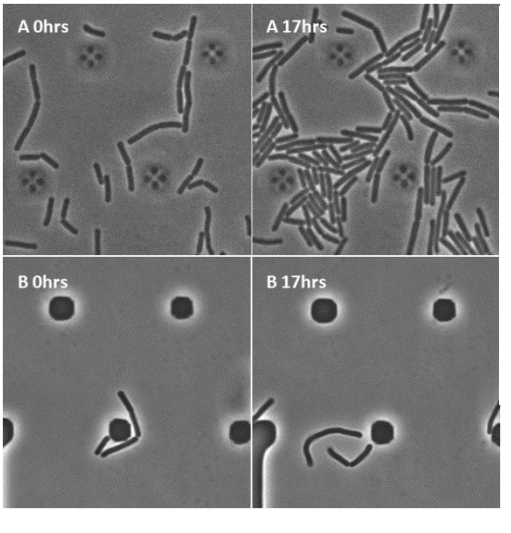
90 minutes after addition of DNA and 60 minutes after the start of the time-lapse foci start disappearing in the samples incubated with DNA. In the control with no added DNA the foci were still visible after 200 minutes of imaging, confirming that foci disappearance is due to the integration of DNA (Img. 1 A & B).



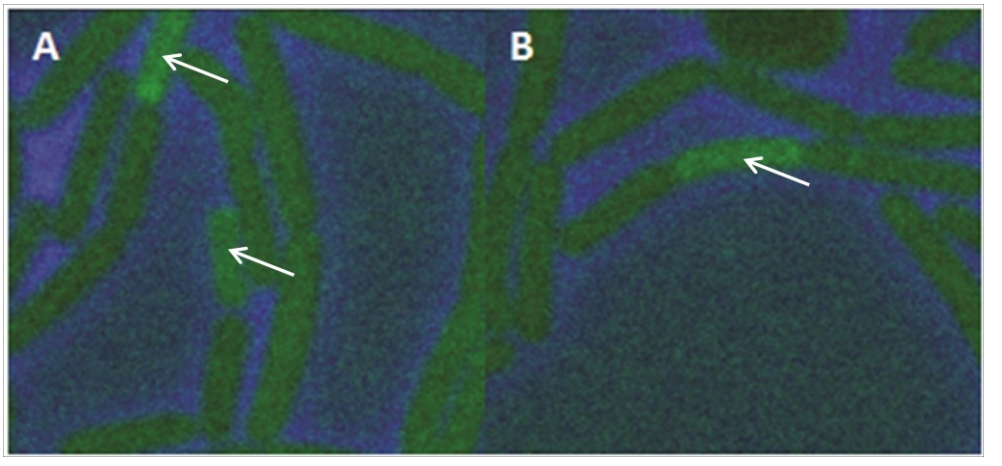
Img. 1 (A).Transformation of *B. subtilis_amyE::P_{xyl}-comK_thrC::parB-mkate2* with PDG1664. The ParB-mKate foci disappear as the *parB-mkate* construct is replaced by PDG1664.
(B).. *B. subtilis_amyE::*P_{xyl} *-comK_thrC::parB-mkate2* without exogenous DNA. The control was performed under the same conditions as the transformed sample but not no DNA was added. The foci are still present after 200 minutes confirming that ParB-mKate remains bound to the *parS* site in the absence of homologous exogenous DNA.

To determine whether labelled DNA can be seen to co-localise with the ParB-mKate foci before replacing them, the same experiment was performed by transformation with fluorescein labelled DNA. Images were taken every minute for 3hrs in order to see co-localisation
In this set up however there is unspecific binding of DNA to the cells, making it more difficult to distinguish individual foci and specific binding (S. Img. 1). Due to rapid imaging combined with high exposure required to overcome the red background of the polyacrylamide slide bleaching occurs and foci disappear in both the transformed sample and the control.

To reduce the bleaching effect the time between imaging was extended to 10 minutes. As with transformation with non-labelled DNA ParB-mKate foci start disappearing 90 minutes after addition of DNA. Unfortunately due to the long time between images (10 min) no-clear co-localisation can be seen (S. Img. 2) Because the high background of the polyacrylamide requires high exposure of mKate fluorescence we chose to switch to a microfluidics system. The advantage is that not only the background is reduced, but we can also more accurately control the conditions. To determine if efficient transformation with labelled DNA occurs in the microfluidics set up we first transformed *B. subtilis* 168_P_{xyl}-*comK* with Dylight650 labelled *thrC_*p_{spank}*-gfp(ery)* DNA. Using a *gfp* construct allows us to determine how long after addition of DNA gene expression occurs and if cell division is required for expression. At all stages of the experiment 1mM IPTG was present in order to induce expression of $P_{hyspank}$-*gfp*. The cells grow in the presence of erythromycin indicating successful transformation (Img. 2A). However, very few cells clearly showed *gfp* expression above the background levels of Bacillus (Img. 4). A control where no DNA is added shows no growth after addition of antibiotics (Img. 2B). The low *gfp* expression could be the result of the strength of the promoter or low oxygen levels in the microfluidics system as GFP requires oxygen for correct folding.

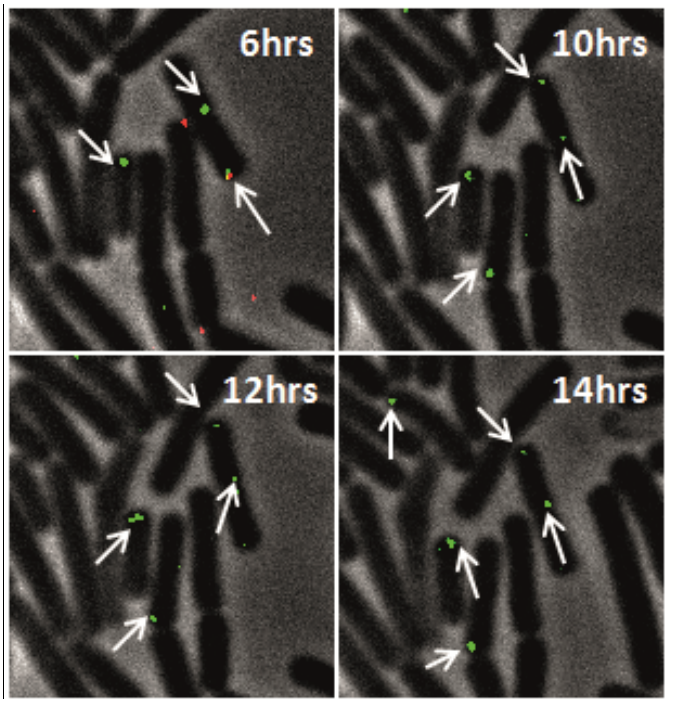


Img. 2 (A). *B. subtilis* 168_*amyE::*P_{xyl}*-comK* transformed with DyLight650 labelled *thrC::*P_{spank}*-gfp(ery)* DNA. Phase contrast image, cells grow in the presence of erythromycin, confirming successful transformation and expression of the erythromycin cassette.**(B). *B. subtilis* 168_*amyE::*P_{xyl}*-comK.*** control where no DNA is added. Cells do not grow in the presence of erythromycin



Img. 3 A & B. *B. subtilis* 168_*amyE::p$_{xyl}$-comK* transformed with DyLight650 labelled *thrC:: P$_{hyspank}$-gfp(ery)* DNA. Expression of the *P$_{hyspank}$-gfp* construct is very low and only a very small number of cells indicated with arrows show clear expression distinguishable from the background fluorescence of *B. subtilis*.

To overcome the problem posed by the low expression of *gfp* and the background fluorescence of *B. subtilis* cells we switched to using a DyLight650 labelled *ThrC_P$_{spank}$-parB-gfp(ery)* construct for transformation. The formation of foci by this construct allows for better distinction between background fluorescence and expression of *gfp*. When transformed with labelled *thrC_P$_{spank}$-parB-gfp (ery)* cells not only divide in the presence of erythromycin, but foci start to become visible 1hr 30minutes after addition of DNA. On average foci became visible after 6hrs and 45minutes. 15% of the cells expressed *gfp* or produced daughter cells expressing *gfp* (Img4. S Table1). Interestingly cell division is not required for expression of *gfp* and about half of the *gfp* expressing cells did not divide before *gfp* was expressed. Not all of the cells dividing after the addition of antibiotics express *gfp* and not all of the cells expressing *gfp* divide after addition of antibiotics 51% of the cells expressing *gfp* also divide. After addition of antibiotics a total of 31% of the cells continue to divide (S. table1), the average doubling time is 61 minutes.



Img. 4. Transformation of *B. subtilis amyE::P_{xyl}-comK* with DyLight650 labelled *thrC::P_{spank}-parB-gfp* DNA. On average foci became visible after 6hrs and 45minutes. 15% of the cells expressed *gfp* or produced daughter cells expressing *gfp*. The arrows indicate a selection of cells expressing *gfp.* Imaging occurred every 15minutes.
Labelled DNA remains visible outside of the cells for several hours as DNaseI is added after 2.1 hrs and levels of DNase do not immediatly reach high enough levels to fuly degrade all DNA outside of the cells
Fresh medium is added after 2hrs, fresh medium with antibiotics is added after 3.1 hrs
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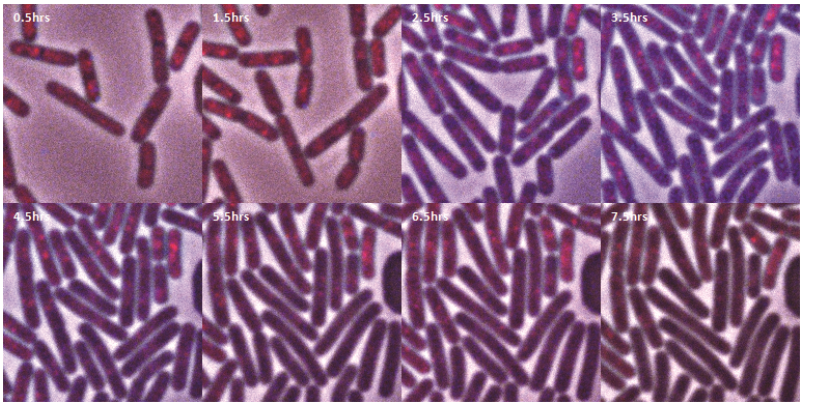
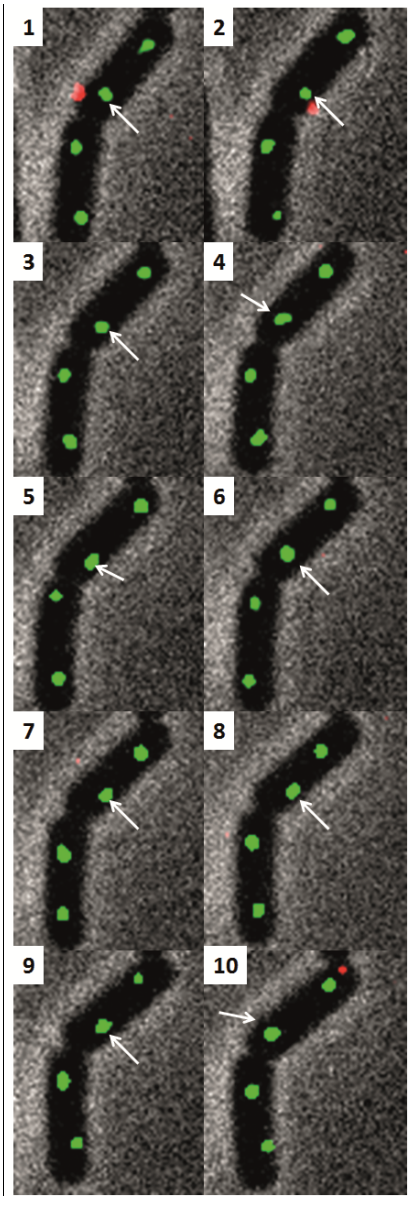
Because co-localisation of fluorescently labelled DNA and foci on the chromosome could not be seen in the experiments on polyacrylamide slides, the experiment was repeated in the microfluidics system with images taken every minute for determining co-localisation and every 15minutes to determine focus displacement (Img.5). To determine if co-localisation with and replacement of the locus by exogenous DNA takes place we transformed *B. subtilis* 168_*amyE::*P_{xyl}*-comK_thrC::*P_{spank}*-parB-gfp(ery)* with DyLight650 labelled *thrC-spec* DNA (from pDG1731) or *B. subtilis* 168_*amyE::*P_{xyl}*-comK_thrC::*P_{spank}*-parB-mkate(ery)* with fluorescein labelled *thrC-spec* DNA. Transforming with homologous DNA containing a spectinomycin cassette allowed us to also select for successful transformation in addition to foci disappearance. As expected, significant bleaching of the ParB-mKate foci does not occur in the microfluidics system due to the lower excitation intensity.

Although uptake of labelled DNA can be seen, significant bleaching does occur between 10-20 excitations of the fluorescein-DNA which made the determination of co-localisation more difficult. Another factor making capture of co-localisation more difficult is the fact that the chromosome is not fixed in one place, but moves around the cell (Img6). Although with the current set-up we were not able to clearly determine co-localisation of a specific locus on the chromosome with labelled DNA, replacement of the foci was observed. Foci disappearance again becomes visible after approximately 90 minutes after addition of DNA. The percentage of cells with no foci increases from 34% at 30min after addition of DNA to 51% after 4.5hrs for the *parB-mkate* strain and from 9% to 41% for the *parB-gfp* strain (S1. tables 2 &3). The percentage of cells containing 2 foci is higher after 4.5 hours of growth. This likely is the result of resumed cell division as *Bacillus* is capable of initiating multiple rounds of replication at once. The inability to clearly distinguish a septum likely also plays a role leading as it can lead to a false foci count. As addition of antibiotics occurs after 3.1hrs, and because it may take some time for the concentration of antibiotics to reach selective concentrations it is possible for cells that do not express the resistance cassette to grow for a while after addition of antibiotics.

The transformed cells grow in the presence of spectinomycin after longer exposure to selective medium confirming successful transformation.
Although cells grow in the presence of spectinomycin, ParB-mkate foci remain visible for several hours. It is possible that instead of replacing the chromosomal construct the exogenous DNA integrates by single cross-over, resulting in the presence of both the spectinomycin and the *parB-mkate* construct in the transformed cells.



Img. 5. Transformation of *B. subtilis* 168_*amyE::P_{xyl}-comK_thrC::P_{spank}-parB-mkate(ery)* with fluorescein labelled *thrC-spec* DNA. As in previous experiment foci begin to disappear after addition of Labelled DNA. Cells continue to grow after addition of spectinomycin. Fresh medium is added after 2hrs, fresh medium with antibiotics is added after 3.1 hrs



Img. 6 Transformation of *B. subtilis amyE::P_{xyl}-comK ThrC::P_{spank}-parB-gfp(ery)* with DyLight650 labelled *thrC-spec* DNA Foci When imaging every minute the green ParB-GFP focus can be seen to move around.

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Discussion

We found that *B. subtilis* is readily transformable in a microfluidics set-up under the conditions tested. The combination of transforming fluorescently-labelled DNA in a microfuidics device and following the process with time-lapse microscopy is an appropriate and convenient method to gain better insight into the transformation process. Using microfluidics instead of a polyacrylamide slide allows for lower intensity and exposure times due to a reduced background fluorescence. The fact that medium actively flows through the system allows for better visualisation of binding and uptake of labelled DNA and the possibility to rapidly change conditions is a great advantage of microfluidics over timelapse microscopy on solid medium slides.

We found that hypercompetent *B. subtilis* can easily be transformed with a relatively high transformation efficiency, as 30% of the cells are able to divide in the presence of the selective antibiotics. The expression of *gfp* originating from the exogenous DNA, however, is lower with 15% of the cells expressing *gfp*. Multiple factors may play a role in the lower expression of *gfp* compared to the resistance gene. The resistance gene is expressed from a constitutive promoter, whereas the *gfp* gene is under the control of a inducible $P_{hyspank}$ promoter. Although the amount of IPTG added to the medium is the maximum (1mM) that is usually used for induction, it is nevertheless possible that the concentration within the microfluidics system might need to be higher as the conditions are different from those within an Erlemeyer flask. The concentration within individual *B. subtilis* cells likely is also different and may in some cells not be high enough for expression. Possible mutations within the *parS* site due to the use of labelled nucleotides may also play a role. Lastly the oxygen concentrations within the microfluidics system could in some cells be too low. Addition of oxygen to the system may stimulate growth and folding of the GFP protein.

In both the solid medium time-lapse and the microfluidics set up, foci start disappearing approximately 90 minutes after addition of DNA.

This is quite a long time especially when taking into account that DNA is taken up rapidly with a speed of 80bp/sec (Maier et al., 2004). This means that our construct should be fully transported after approximately 31 seconds.
Due to technical limitations we were not able to clearly visualise co-localisation with a specific locus and were unable to determine how long it takes for the recombination system to find the homologous region and begin strand invasion. A possible reason for the long time before foci disappearance is that the ParB in our construct is able to bind single stranded DNA during recombination and therefore is not completely displaced during recombination. Also, initially two different strands are present: one with the integrated DNA and one with the original ParB construct. The *Bacillus* ParB (Spo0J) is able to bind single stranded DNA Cervin et al., 1998, Marston and Errington, 1999, Quisel et al., 1999 and Quisel and Grossman, 2000), and it is therefore likely that the ParB in our construct is able to as well. It also takes at least 90 minutes before expression of *gfp* from the integrated DNA first becomes visible with the average time for expression of GFP being 6hours and 45 minutes after addition of DNA under these conditions. In *E.coli* during exponential growth the rate of transcription is 10-100nt/sec and the speed of translation 10-20 aa/sec (Milo & Philips 2014). Transcription and translation of *gfp* will therefore take at minimum 8-80sec and 12 - 24sec respectively.
Even though the bacterium is in the competent state, where RNA synthesis is reduced (Dooley et al., 1971), it is likely that transcription and translation are not the limiting factors. Competent cells need to exit competence before resuming replication and cell-division. Halting of cell division and replication gives the bacteria time to integrate exogenous DNA before resuming growth. It is therefore most probable that the relatively long time before expression is primarily the result of the recombination and repair process. Haijema et al. found that competent cells resumed replication and growth 2-3 hrs after dilution in fresh medium (Haijema et al., 2001). Under our conditions the average time of *gfp* expression is 6.45 hours after addition of DNA and 4.45 hours after addition of fresh medium.

The average doubling time in this set-up at 60 minutes is approximately double that of the division time in a shake flask. This timing may indicate that the expression of exogenous DNA coincides with resumed replication. It would be interesting to determine if replication is indeed required before expression of the integrated DNA. Replication might be needed for expression of exogenous DNA, as the mismatch repair proteins MutS and MutL localise at mismatches that emerge from DNA polymerase (Smith et al., 2001). It has been found that mutations in *mutSL* of *Streptococcus pneumoniae* reduces transformation efficiency, when exogenous DNA has approximately 5% difference in sequence compared to the recipient (Mortier-Barriere et al., 1997). It is therefore likely that for successful expression mismatches need to be repaired. In this study we found that although division occurs in half of the cells expressing *gfp,* division is not required for expression. Expression of exogenous before division is beneficial as many antibiotics are capable of inhibiting cell division or killing cells during division by inhibiting cell wall synthesis. More generally if transformation is primarily a method of obtaining genes that improve fitness, being able to express genes that provide a fitness benefit before division makes sense, as division is an energy intensive process. Despite the technical limitations, we have shown that *B. subtilis* is able to successfully take up and integrate labelled DNA in a microfluidics setting. The fluorescently labelled DNA can be seen to replace a specific locus on the chromosome by replacing the *parB-mkate/parB-gfp* construct on the chromosome. We have also shown that cell-division is not required for expression of the integrated DNA under these conditions. The primary technical limitation is bleaching of the fluorescent dye. Use of a more photostable dye should allow for better results. In particular it will enable visualisation of interaction between the foci on the chromosome and the labelled DNA. For future research it would be interesting to create a system in which the labelled-DNA encoding a fluorescent protein is transformed into a strain with protein fusions of a component of the competence machinery, a recombination protein such as RecA a visualised chromosomal locus and a replication marker, thereby following the entire process.

Experimental procedures

Strain construction

B. subtilis 168_*amyE:: P_{xyl}-comK _thrC::*P_{spank} *parB-gfp* and *B. subtilis* 168_*amyE:: P_{xyl}-comK thrC::P_{spank} parB-mkate2* were created by amplification of *parB-mkate2* from pMK17 and *parB-gfp* from pMK11 (Kjos et al., 2015; Raaphorst et al., 2016) with primers 133 and 134. *parB-gfp* and *parB-mkate* were cloned into pMB002 using NheI and HindIII (FastDigest Thermo Scientific) ligated with T4 ligase (Thermo Scientific) and transformed into *E.coli* DH5-α and sequenced. *B. subtilis 168 amyE:: P_{xyl}-comK* was transformed with pMB002-parB-mkate2 or pMB002-parB-gfp. PDG1664 *$P_{hyspank}$-gfp* used for transformation was created by Dr Anne-Stephanie Rueff.

Strain	Genomic context	Reference
B. subtilis 168 p_{xyl}-*comK*	*amyE::*p_{xylR}-p_{xylA}-*comK*, *trpC2* cm^r	(Hahn et al., 1996)
B. subtilis 168 P_{xyl}-*comK*_P_{spank}-*parB-gfp*	*amyE::* p_{xyl}*R-*p_{xylA}*-comK_ thrC::*P_{spank} *parB-gfp, trpC2* $cm^r ery^r$	
B. subtilis 168 p_{xyl}-*comK_parB-mkate2*	*amyE::* p_{xyl}*R-*p_{xylA}*-comK_ thrC::*P_{spank} *parB-mkate2, trpC2* $cm^r ery^r$	

Primers

ID	name	sequence
prMB013	PDG1664-ery_F	GGGAACGGTTGGAGCTAATG
prMB014	PDG1664-ery-R	TTCCGGGAACAGTGACAGAG
133		GATCAAGCTTGAGTACTGATTAACTAATAAGGAG
134		TACTAGCTAGCGCTATCAAAAGAATCTTGC

Growth conditions

For the competence experiments a medium adapted from (Spizizen 1958) and (Konkol et al., 2013) 18ml demi water, 2ml 10X competence medium stock (0.615M K_2HPO_4 . $3H_2O$, 0.385M KH_2PO_4, 20% gluocose, 10ml 300mM Tri-Na-citrate, 1ml 2% ferric NH_4 citrate, 1g casein hydrolysate (Oxoid), 2g potassium glutamate) 100µl 2mg/ml tryptophan, 67µl 1M $MgSO_4$. where indicated glucose was replaced by fructose as carbon source. Strains were grown in 20 ml of competence medium. For these experiments the following conditions were used. A single colony grown overnight on LB agar plate containing 5µg/ml chloramphenicol and 12.5 µg/ml lincomycin + 0.5 µg/ml erythromycin in the case of the P_{spank}-*parB-gfp* and Ps_{pank}-*parB*-mkate strains at 37C was diluted 10^3X-10^5 in PBS or 1X Spizizen solution to ensure that the cultures are in the exponential growth phase after overnight growth. 100µl of the diluted sc colony solution was added to 20ml medium 5µg/ml chloramphenicol and 12.5 µg/ml lincomycin + 0.5 µg/ml erythromycin in the case of the P_{spank}-*parB-gfp* and P_{spank}-*parB-mkate* strains in 100 ml Erlenmeyer flasks and grown at 37 Celsius 220rpm.

The overnight cultures were diluted to an OD600 of 0.05 in 20ml medium without antibiotics. The P_{xyl}-*comK* strains were induced with 0.5% xylose after 4hrs of growth. The P_{spank}-*parB* strains were also induced with 1mM of IPTG after 4hrs of growth.

PCR reaction for labelling with Fluorescein

1µl of 1mM fluorescein-12-dUTP (Thermo Fisher Scientific) and 2µl dNTP mix (1mM dATP, dCTP, dGTP, 0.5mM dTTP (Thermo Fisher Scientific), 0.5 µl DreamTaq DNA polymerase (Thermo Fisher Scientific) 5µl DreamTaq buffer, 1µM prMB013 and 1µM prMB014, 100ng template total volume 50µl. 35 cycles of a standard Dreamtaq PCR protocol was used. A longer extension time of 3min was used for a 2300bp product. After PCR samples were incubated for 2hrs with 0.5µl DpnI (FastDigest Thermo Fisher Scientific).PCR samples were purified using a Machery-Nagel PCR kit. Samples were stored at -20 ^{0}C.

Samples were protected from light at all times. The label incorporation of Fluorescein-dUTP lies between 1-3 pmol measured by nanodrop.

Labelling with DyLight650
1µl dNTP mix (10mM dGTP, dCTP, dATP, 5mM dTTP, 5mM aminoallyl-dUTP (Thermo Fisher Scientific) 0.25 µl Dreamtaq (Thermo Fisher Scientific 5µl Dreamtaq buffer, 1µM prMB013 and 1µM prMB014, 100ng template. Total volume 50µl, to obtain a high enough amount of product 8 50µl reactions are needed. The PCR program was the same as for Fluorescein. Samples were incubated for 2hrs with 0.5µl DpnI (FastDigest Thermo Fisher Scientific). Samples were purified with a Machery-Nagel PCR kit PCR kit The second wash step was done with 80% ethanol and the samples were eluted with 60µl 0.1M $NaHCO_3$ pH 9. Samples were incubated for 3hrs with Dylight650. Samples were purified with a Machery-Nagel PCR kit. Labelling resulted in an incorporation of 1-2 pmol as measured by Nanodrop.

Time lapse on polyacrylamide slides
Cells were immobilised by polyacrylamide. Polyacrylamide slides were made with 500µl 40% acrylamide, 1.5ml 1X competence medium, 20µl 10% ammonium persulphate and 2µl TEMED for the strains containing Pspank-parB constructs 1mM of IPTG was added. A gene frame (Thermo Fisher Scientific or Westburg) was stuck on a glass object carrier and the polyacrylamide was added and covered with another object carrier. The slide was left to solidify after which the top slide was removed and the solidified gel was washed 3x 30 minutes with sterile double distilled water. The gel was kept in competence medium containing IPTG for experiments using P_{spank}-*parB* constructs until needed and cut in smaller pieces when necessary. 100 µL of cells were collected by centrifugation at 11000rcf for 3 min. Supernatant was removed and the pellet was resuspended in 20 µl of PBS. DNA was added to a final concentration of 2.5 ng/µl. 0.5 µl of cells were added to the pre-prepared polyacrylamide slide. Microscopy was performed on a GE-healthcare OlympusIX71DV.

The following setting were used for timelapse microscopy on polyacrylamide slides. A 100x oil-immersion objective was used.
Settings for time-lapse were: Phase contrast 200 ms excitation, intensity 32%. The mCherry Filter was used for excitation of mKate2 excitation 1 second, intensity 50%.
When using fluorescein-labelled DNA, the GFP filter was used with excitation 250-300 ms and intensity 5 to 10%. Total time for time lapse was from 4 to 6 hours, time interval of taking pictures was 10 min or 1 minute for determining co-localistation localization with foci. Images were acquired and deconvolved using Softworx (Applied Precision) and analyzed using ImageJ or Fiji (http://fiji.sc/Fiji). Images were put to a 300 pixels/inch CMYK format with Adobe photoshop.

Microfluidics experiments
Cultures were grown in glucose containing competence medium as for the polyacrilamide time lapse. After 4hrs .5% xylose and 1mM IPTG for the P_{spank}-*parB* constructs. After 5hrs 2ml of the culture was spun down at maximum rpm on a standard table top centrifuge and the supernatant was filtered with a Puradisc FP30mm 0.45 μm filter. After 5hrs of growth the culture was diluted 50X in filtered supernatant. The experiments were performed with the CellASIC ONIX Microfluidic (Merck Millipore) device in a BO4A plate. The plate was primed with the media before the start of the experiment using the manufacturer's specifications. The samples were loaded according to the manufacturer's specifications. The following media and growth conditions were used: step1 incubation with 2.5ng/ul DNA in 100μl supernatant for 2hrs. Step2: incubation with fresh media containing 10U of DNaseI for 10minutes. Step 3: incubation with fresh medium for 1hr. Step4: Incubation for 16-20hrs with fresh medium containing selection antibiotics. For experiments with the P_{spank}-*parB* and $P_{hyspank}$-*gfp* strains 1mM of IPTG was present each medium. The flow rate was 0.25. Experiments were performed at 37°C.

The exposure times were 0.8seconds intensity 32% (mCherry filter) for mKate2. 0.8second 32% intensity for GFP (GFP filter), 0.5seconds 32% for fluorescein-DNA (FITC filter), 0.5seconds 32% for Dylight650 DNA (Cy5 filter). The images were deconvolved with the Softworks sofware. Analysis was done using ImageJ.

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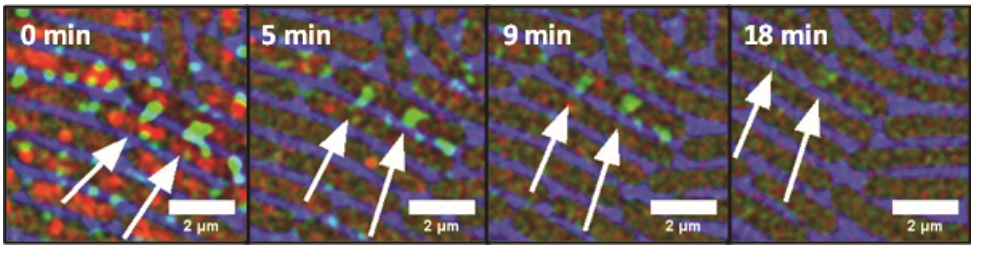
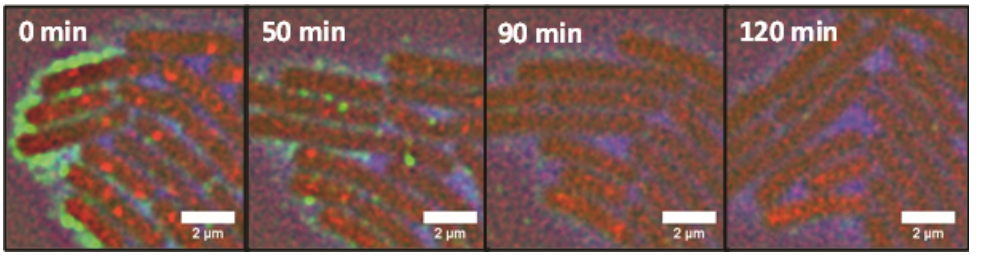
Supplementary material Chapter 5

Images



Img. 1: Transformation of *B.subtilis* 168_*amyE::pxyl-comK*_*ThrC::parB-mkate2* with fluorescein-labelled *thrC-ery* DNA. Images were taken ever minute in order to determine co-localisation. The arrows indicate possible co-localisation of fluorescein-DNA with ParB-mKate. Unfortunately the high exposure conditions result in rapid bleaching of both the labelled DNA and the ParB-mKate foci. The presence of apparent unspecific binding and and the use of the polyacrylamide slide requiring high exposure makes it more difficult to accurately determine co-localisation.



Img. 2 Transformation of *B. subtilis* 168_*amyE::pxyl-comK*_*ThrC::parB-mkate2* with fluorescein-labelled *thrC-ery* DNA. Imaging every 10 minutes. As with transformation with non-labelled DNA ParB-mkate foci start disappearing 90minutes after addition of DNA. Unfortunately due to the long time between images (10min) no-clear co-localisation can be seen

start nr. cells	194
tot. nr. cells at addition of antibiotics	267
% of cels dividing after addition of antibiotics	31
% of cells showing foci	15

Table 1 Cells dividing and/or showing foci after transformation with P_{spank}-*parB-gfp*. Not all of the cells dividing after addition of antibiotics express *gfp*, and not all of the cells expressing *gfp* divide after addition of erythromycin. 51% of the cells expressing GFP also divide.

ParB-mKate	30min	1.5hrs	2.5hrs	3.5hrs	4.5hrs
tot. nr. cells	211	375	483	669	729
percentage 1 focus	53	44	39	33	27
percentage 2 foci	13	10	10	14	22
percentage no foci	34	47	52	53	51

Table 2 Disappearance of ParB-mKate foci of *B. subtilis amyE::P_{xyl}-comK ThrC::P_{spank}-parB-mkate(ery)* after addition of Fluorescein labelled *thrC-spec* DNA. The percentage of cells with no-foci increases over time.

ParB-GFP	30min	1.5hrs	2.5hrs	3.5hrs	4.5hrs
tot. nr. cells	211	217	282	421	547
percentage 1 focus	72	62	69	44	23
percentage 2 foci	20	24	9	27	36
percentage no foci	9	14	22	44	41

Table 3. Disappearance of ParB-GFP foci of *B. subtilis amyE::P_{xyl}-comK ThrC::P_{spank}-parB-gfp(ery)* after addition of DyLight650 labelled *thrC-spec* DNA. The percentage of cells with no-foci increases over time.